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Total number of alterations in liquid biopsies is an independent predictor of survival in patients with advanced cancers

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Running Head: Total number of alterations in liquid biopsies is prognostic

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Conflicts of Interest:

Dr. Razelle Kurzrock has research funding from Incyte, Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, and Guardant Health, as well as consultant fees from Sequenom, Loxo and Actuate Therapeutics, speaker fees from Roche, and an ownership interest in IdbyDNA and Curematch, Inc

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Context Summary:

Key objective: Can we utilize liquid biopsies to obtain prognostic information for patients with advanced cancers?

Knowledge generated: We demonstrate that an increasing number of genomic alterations found on liquid biopsy correlates with progressively worse survival in patients with gastrointestinal and other advanced cancers, independent of the **percent** ctDNA or allele fraction.

Relevance: The total number of alterations found on a liquid biopsy may be a marker of more aggressive tumor biology and has the potential to become a clinically meaningful, tissue-

agnostic biomarker for use in advanced cancers and warrants further testing in a prospective manner.

Revised December 23, 2019

ABSTRACT

PURPOSE: Studies have demonstrated an association between quantity of circulating tumor DNA (ctDNA) and poorer survival. We investigated the relationship between %ctDNA, total number of ctDNA alterations, and overall survival (OS) in liquid biopsies.

PATIENTS AND METHODS: Overall, 418 patients with blood-based next generation sequencing (54 to 73 genes: Guardant Health) were analyzed. Eligible patients included those who had solid tumor malignancies and never received immunotherapy treatment, which may alter the survival curve in patients with high mutational burden.

RESULTS: Patients with a high ($\geq 5\%$) %ctDNA had significantly shorter OS versus those with intermediate ($\geq 0.4\%$ to $< 5\%$) or low ($< 0.4\%$) values (median OS, 7.0 vs. 14.1 vs. not reached (NR) months, respectively; $P < .0001$). Patients with a high (≥ 5) total number of alterations had significantly shorter OS versus those with intermediate (≥ 1.46 to < 5), low (< 1.46), or no alterations (median OS 4.6 vs. 11.7 vs. 21.3 vs. NR months, respectively; $P < .0001$). The total number of alterations correlated with %ctDNA ($r = 0.85$; 95% CI, 0.81-0.87; $P < 0.0001$). However, only an intermediate to high total number of alterations (≥ 1.46) was an independent predictor of worse OS (hazard ratio 1.96; 95% CI, 1.30-2.96; $P = 0.0014$; multivariate analysis).

CONCLUSION: We demonstrate that the total number of alterations and %ctDNA have prognostic value and correlate with one another, but only the total number of alterations was independently associated with survival outcomes. Our findings suggest that the total

number of alterations in plasma may be an indicator of more aggressive tumor biology and therefore poorer survival.

INTRODUCTION

Five-year survival rates are incredibly variable among cancer types, ranging from over 90% in prostate cancer to less than 8% in pancreas cancer, and depend heavily on clinical and pathologic stage ¹. Although repeat tissue biopsies during the course of treatment or at the time of progression may provide clinically important information, such biopsies are not routinely performed because they can be technically difficult, time consuming, medically invasive, and lead to complications. On the other hand, liquid biopsies, or cell-free DNA (cfDNA) obtained from blood plasma that contains fragments of circulating tumor DNA (ctDNA) shed from tumor cells into the bloodstream, can identify new actionable alterations and be performed repeatedly with minimal procedural risk ²⁻⁵. ctDNA can then be analyzed using technologies such as digital polymerase chain reaction (PCR) to detect specific known somatic variants (e.g., *EGFR* T790M) or next-generation sequencing (NGS) that uses massive parallel sequencing to detect up to thousands of somatic and germline alterations in a single run ⁶. In addition, genomic alterations found on liquid biopsies are often concordant with alterations found on tissue biopsy when obtained within close proximity to one another ⁷⁻⁹.

A number of studies have demonstrated that there is an association between higher amounts of cfDNA or ctDNA and poorer survival, perhaps because percent ctDNA (%ctDNA) correlates with tumor burden ^{10, 11}. For the most part, these reports dichotomized the level of cfDNA or ctDNA at a cut-point (often but not always at ~5% or 10% ctDNA) ^{10, 12-18}. In the case of surgical candidates, the cut-points may be lower. For instance, Baumgartner and colleagues found that pre-operative levels of %ctDNA $\geq 0.25\%$ in patients with peritoneal carcinomatosis were an independent predictor of shorter progression-free survival ¹⁵.

In the current study, we sought to more comprehensively examine the relationship between %ctDNA versus the total number of alterations found in liquid biopsies and outcome.

MATERIALS AND METHODS

Patient Data

Overall, 418 consecutive eligible patients at the University of California San Diego who had NGS (54 to 73 genes: Guardant Health) performed on ctDNA derived from liquid (blood) biopsies were analyzed. Eligible patients included those who had solid tumor malignancies, never received immunotherapy treatment, and were evaluable for clinical correlations including overall survival (OS) from ctDNA collection date. Patients had advanced/metastatic (stage IV) disease (except for patients with CNS tumors) at the time of ctDNA analysis. Immunotherapy-treated patients were omitted because a correlation with blood or tissue tumor mutational burden has been associated with better immunotherapy response and might therefore alter the survival curve^{19, 20}. Patients with amplifications only in ctDNA were omitted because the %ctDNA for amplifications could not be determined. In addition to OS evaluation, patients' data was also collected and analyzed for %ctDNA (the alteration with the highest allele fraction was calculated from all alterations, including variants of unknown significance (VUS)), total number of VUSs, and total number of alterations (which included VUSs). Percent ctDNA was evaluated as a continuous variable as well as using a cut-point of $\geq 5\%$, as this threshold had been found to be significant in prior studies¹⁰. All studies and analyses were performed in accordance with the ethical guidelines of the Declaration of Helsinki and the Belmont Report per a University of California San Diego, Internal Review Board-approved protocol (NCT02478931) and the investigational treatment protocols for which the patients gave written consent. All human investigations were performed after approval by a local Human Investigations Committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services.

ctDNA Sequencing

Sequencing was performed by a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory, Guardant Health, Inc. (<http://www.guardanthealth.com>). The Guardant360 (54 to 73 gene) panel identifies characterized and VUS tumor-related genomic alterations within cancer-related genes. All values for the total number of ctDNA alterations and the number of VUSs were corrected for the length (kilobase pairs, kbp) of DNA sequenced based on the date sequencing was performed and multiplied by 100 (**Supplemental Table 1**). All data was analyzed from the time of ctDNA collection from plasma (two 10-mL blood tubes). This ctDNA assay has a sensitivity and specificity of >85% and >99.9999%, respectively, for detection of single-nucleotide variants in tumor tissue of advanced cancer patients ²¹.

Statistical analysis:

Statistical analysis was performed by PR. Hazard ratios (HR) for survival were calculated by comparing OS above and below cutoffs and performed from the time of ctDNA collection; dichotomization for each variable (i.e., total number of alterations, total number of VUSs, %ctDNA) was performed at the median. Survival analyses were calculated by Kaplan-Meier analysis using log-rank (Mantel-Cox) test to generate p-values, hazard ratios, and confidence intervals (CI). Linear regressions were performed using the least squares method. Multivariate analyses were conducted using the Wald chi-square test from a Cox proportional hazards model that included all variables with $P \leq 0.05$ in univariate analyses (i.e., gender, age, total number of alterations, %ctDNA), with the exception of VUSs because these alterations are already encompassed within the total number of alterations variable. Patients alive at the time of last follow up were censored at that date. Associations between %ctDNA and total number of alterations were determined using Spearman's rank-order correlation. Bootstrapping utilizing

random sampling with replacement ($N = 1,000$ bootstrap samples) and multiple logistic regression analysis was performed, permitting the data of the sample study to be used as a surrogate for a larger population to validate the model. This method can be used when the sample size is too small to be split into training and validation sets and there is no independent cross-validation cohort, as is the case in our study ²². Statistical analyses were carried out using Graph-Pad Prism version 7.0 (San Diego, CA, USA) and R version 3.5 (R Foundation for Statistical Computing, Vienna, Austria, <http://www.R-project.org/>).

RESULTS

Patient Characteristics:

This study included 418 patients who had NGS performed on plasma-derived ctDNA and did not receive immunotherapy treatment. The median age at diagnosis was 60 years (range, 14-92) and the number of men (n=191/418; 46%) and women (n=227/418; 54%) were balanced. The most common tumor types included gastrointestinal (GI) (n=173/418; 41.4%), thoracic (n=94/418; 22.5%), central nervous system (n=51/418; 12.2%), and others (n=100/418; 23.9%) (**Table 1**). After correcting for the kbp length of DNA sequenced for each sample, the median total number of ctDNA alterations (including VUSs) per patient was 1.46 (range, 0-78.8); the median total number of VUS alterations per patient was 0.66 (range, 0-64.2); and the median %ctDNA was 0.4% (range, 0-80.3%) (**Table 1**). Among patients with GI tumors, the median total number of ctDNA alterations was 1.46 (range, 0-78.8) and the median %ctDNA was 0.5% (range, 0-75%).

Factors Correlating with Survival in Univariate Analysis

The following factors showed significant correlations with poorer survival in univariate analysis: gender, older age (dichotomized at the median of 60 years), higher total number of alterations/kbp DNA (dichotomized at the median of 1.46), greater number of VUS alterations/kbp DNA (dichotomized at the median of 0.66), and higher %ctDNA (dichotomized at the median of 0.4%) (**Table 1**). Tumor organ of origin was not found to be significantly correlated with differences in survival.

Patients with %ctDNA greater than or equal to the median of 0.4% had inferior OS compared to those with less than 0.4% (HR, 2.04; 95% CI, 1.48-2.83; $P<0.0001$) (**Table 1**). Furthermore, patients with a high %ctDNA ($\geq 5\%$) had a significantly shorter OS

compared to those who had an intermediate ($\geq 0.4\%$ to $< 5\%$) or low ($< 0.4\%$) value (median OS 7.0 vs. 14.1 vs. not reached months, respectively; $P < .0001$) (**Figure 1**). Among patients with GI tumors, those with %ctDNA greater than or equal to the GI median of 0.5% had worse survival outcomes (HR, 2.46; 95% CI, 1.50-4.03; $P < .0001$; **Table 2**).

Likewise, patients with ≥ 1.46 total alterations/kbp DNA had statistically inferior OS compared to those who had less than the median of 1.46 total alterations/kbp DNA (HR, 2.42; 95% CI, 1.75-3.36; $P < 0.0001$) (**Table 1**). Patients with a high (≥ 5) total number of alterations/kbp DNA had significantly shorter OS compared to those who had an intermediate (≥ 1.46 to < 5), low (< 1.46), or no alterations (median OS 4.6 vs. 11.7 vs. 21.3 vs. not reached months, respectively; $P < .0001$) (**Figure 2**). In the subset of patients with GI tumors, patients with greater than or equal to the median of 1.46 total alterations/kbp had worse survival outcomes (HR, 3.46; 95% CI, 2.09-5.72; $P < 0.0001$; **Table 2**). Also, a higher number of VUS alterations/kbp DNA (≥ 0.66) was associated with worse OS compared to those with a lower number (< 0.66) of VUS alterations/kbp (HR, 1.69; 95% CI, 1.24-2.30) (**Table 1, Supplemental Figure 1**).

Correlation between %ctDNA and total number of alterations

The following ctDNA variables showed significant correlations with one another: the %ctDNA and the total number of alterations tend to increase together ($r = 0.85$; 95% CI, 0.81-0.87; $P < 0.0001$) (**Figure 3**) and the number of VUS alterations and total number of alterations tend to increase together ($r = 0.73$; 95% CI, 0.68-0.77; $P < 0.0001$) (**Supplemental Figure 2**). To evaluate the influence of patients who had no detectable alterations ($n = 112$), we performed a sensitivity analysis removing these patients from the correlation calculations and found that there was still a significant (albeit attenuated)

correlation between %ctDNA and total number of alterations ($r = 0.61$, $p < 0.0001$) as well as VUS and total number of alterations ($r = 0.60$, $p < 0.0001$).

Factors Correlating with Survival in Multivariate Analysis

After accounting for gender, age, total number of alterations, and %ctDNA, a multivariable Cox proportional hazard regression model showed that age, sex, and the total number of alterations were independently prognostic for survival (**Table 1**). Specifically, patients with a high number of alterations (≥ 1.46) compared to those with fewer alterations (< 1.46) had worse OS (HR 1.96; 95% CI, 1.30-2.96; $P = 0.0014$). Although statistically significant in univariate analysis, higher %ctDNA ($\geq 0.4\%$) was not predictive of poorer survival compared to those with lower %ctDNA ($< 0.4\%$) (HR, 1.31; 95% CI, 0.87-1.97; $P = 0.19$) in multivariate analysis (**Table 1**). We also analyzed the subset of patients with GI tumors and found similar results. Although univariate analyses of the GI subset of patients showed that both a high number of alterations (≥ 1.46) and higher %ctDNA ($\geq 0.5\%$) had prognostic value, only the total number of alterations (HR, 3.23; 95% CI, 1.73-6.03; $P < 0.0001$), not the %ctDNA (HR, 1.36; 95% CI, 0.73-2.51; $P = 0.34$), was associated with worse outcomes in the multivariate model (**Table 2**).

Analysis with bootstrapping method

Bootstrapping with multiple logistic regression was performed on all variables with $P \leq 0.05$ in univariate analysis, which included sex, age, total number of alterations, and %ctDNA. Among these characteristics, only total number of alterations was significantly associated with survival ($P < 0.0001$; **Table 1**).

DISCUSSION

Liquid biopsies have been incorporated into clinical practice as a means to obtain noninvasive molecular profiling in order to identify specific oncogenic driver mutations or other alterations that can guide treatment selection. In this study, we evaluated the relationship between the total number of alterations and the %ctDNA detected by liquid biopsy and survival outcomes in 418 patients with advanced cancers. The objective was to explore the potential prognostic value of blood-based NGS. It should be noted that we adjusted for changes in sequencing length by correcting the total number of alterations and VUSs for the amount of DNA sequenced. In addition, we intentionally excluded patients who subsequently received immunotherapy treatment, as several studies have suggested that the use of immune checkpoint inhibitors may alter the survival curve in patients with increased tumor mutational burden ^{19, 20, 23}.

We demonstrate that both the total number of alterations and the %ctDNA have prognostic value and correlate with one another, but only an intermediate to high (≥ 1.46) total number of alterations/kbp (and not high %ctDNA) was independently associated with worse survival outcomes in multivariate analysis in patients with GI tumors (**Table 2**) as well as in patients with a diverse group of advanced cancers (**Table 1**). These findings were then internally validated using bootstrap resampling. Our findings suggest that more alterations per kbp DNA detected in plasma may be a better indicator of more aggressive tumor biology and therefore poorer survival than %ctDNA. It is also plausible that the higher number of alterations and accompanying aggressive biology results in a higher tumor burden that yields a higher %ctDNA (rather than vice versa).

A strength of our study is that we utilized sequencing technology that allows for the detection of %ctDNA at a low level with high sensitivity and very high specificity ^{21, 24}. In comparison, some prior studies have utilized low-depth sequencing, which is less

capable at detecting ctDNA. As a result, these studies were only able to conclude that the presence of ctDNA was associated with worse outcomes compared to the absence of detectable ctDNA^{14, 18, 25, 26}. Indeed, Yang *et al* proposed that the presence or absence of ctDNA should be added to the TNM staging classification of tumors because it has diagnostic, therapeutic, and prognostic value²⁷. When greater depth of ctDNA sequencing was used, prior studies have reported that %ctDNA is correlated with worse survival and also with increased tumor volume^{10, 11, 14}. We also demonstrated that %ctDNA correlates with survival measured from the time of blood draw (**Figure 1**), which suggests that the association between %ctDNA and outcomes may be more reflective of tumor burden.

There are several limitations to our findings given the retrospective nature of the analysis. Although our study utilized a relatively large sample of 418 patients, we included a diverse group of advanced cancers and, therefore, our findings may not be applicable to certain tumor types. On the other hand, the variety of tumor types in our study may make our findings more generalizable across advanced cancers. However, we also performed the analyses a cohort of 173 patients with gastrointestinal tumors and found similar results (**Table 2**). In addition, 112 patients in this study had no detectable %ctDNA, which may be due to low disease burden or due to limitations of the ctDNA sequencing technique. It should also be noted that there were different number of subgroups in the analysis of %ctDNA and number of ctDNA alterations; hence the conclusion that the total number of alterations and %ctDNA have prognostic value and correlate with one another, but only the total number of alterations was independently associated with survival outcomes will need to be further examined and validated. Also, we do not know if this patient population is comparable to those who were not analyzed for ctDNA because physicians chose not to perform the analysis, or to patients who were

lost to follow up early and hence were inevaluable. Finally, patients had a diverse array of prior treatments, some of which could have confounded the results; patients treated with immunotherapy were excluded because cancers with higher mutational burden/number appear to do better on this modality.

In conclusion, to our knowledge, this is the first demonstration that the total number of alterations and %ctDNA are highly correlated and have prognostic value. Nevertheless, in multivariate analysis, only the total number of alterations was independently predictive of overall survival. Understanding the prognostic value of ctDNA is important in and of itself, but also has implications as a confounder, since ctDNA is being utilized as a predictive marker for the efficacy of drugs such as immunotherapy ²⁰. To summarize, the total number of alterations has the potential to become a clinically meaningful, tissue-agnostic biomarker for use in advanced cancers and warrants further testing in a prospective manner.

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Table 1: Univariate and multivariate analyses of patient and ctDNA characteristics on survival (n = 418)^a

Variable	Group	N=418 (%)	Median OS (months)	HR (95% CI) Univariate ^e	P value Univariate	HR (95% CI) Multivariate ^f	P value Multivariate ^f	P value Bootstrap ^h
Sex	Women	227 (54%)	79	Reference Group	0.01	Reference Group	0.04	0.1
	Men	191 (46%)	53	1.47 (1.08 - 2.00)		1.39 (1.02 - 1.89)		
Age	≤60 years	225 (54%)	76.6	Reference Group	6.1x10 ⁻⁴	Reference Group	0.01	0.26
	>60 years	193 (46%)	50.4	1.72 (1.26 - 2.34)		1.50 (1.09 - 2.05)		
Tumor Type	Gastrointestinal	173 (41%)	54.6	1.59 (1.08 - 2.34)	0.12	Omitted		
	Thoracic	94 (22%)	72.7	1.28 (0.80 - 2.03)				
	CNS	51 (12%)	61.4	1.15 (0.69 - 1.94)				
	Other ^d	100 (24%)	87.1	Reference Group				
(Total Alterations x 100 / kbp) ^b	<1.46	203 (49%)	104.8	Reference Group	1.1x10 ⁻⁷	Reference Group	1.4x10 ⁻³	1.8x10 ⁻⁵
	≥1.46	215 (51%)	42.2	2.42 (1.75 - 3.36)		1.96 (1.30 - 2.96)		

(VUS x 100 / kbp) ^b	<0.66	207 (50%)	87	Reference Group	8.9x10⁻⁴	Omitted ^g		
	≥0.66	211 (50%)	53	1.69 (1.24 - 2.30)				
%ctDNA ^c	<0.4	195 (47%)	105	Reference Group	1.6x10⁻⁵	Reference Group	0.19	0.11
	≥0.4	223 (53%)	50	2.04 (1.48 - 2.83)		1.31 (0.87 - 1.97)		

Note: All survival analyses were performed from the time of ctDNA collection

Abbreviations: CI = confidence interval; CNS = central nervous system; ctDNA = circulating tumor DNA; %ctDNA = percent circulating tumor DNA; HR = hazard ratio; kbp = kilobase pairs; NR = not reached; OS = overall survival; VUS = variants of unknown significance

^a Patients treated with immunotherapy were excluded

^b Dichotomization done at medians

^c Dichotomization done at medians of highest %ctDNA for each patient; alteration with highest %ctDNA was calculated from all alterations including VUSs. Patients with only amplifications were considered inevaluable and excluded. Kbp indicates the length of ctDNA sequenced: (See **Methods**)

^d Other Tumor Type includes Breast (n=29), Head and Neck (n=21), Genitourinary (n=13), Gynecologic (n=18), Neuroendocrine, Sarcoma, Melanoma, and Unknown Primary

^e Hazard Ratio performed using log-rank

^f Variables with $P \leq 0.05$ in univariate analysis were included in the multivariate analysis. Separate analyses were done to include %ctDNA, total alterations

^g VUS was excluded from the multivariate analysis because it is encompassed within total alterations

^h Bootstrapping with multiple logistic regression analysis was conducted on characteristics with $P \leq 0.05$ in univariate analysis. P-value based on 1,000 bootstrap samples.

Table 2: Univariate and multivariate analyses of patient and ctDNA characteristics and survival for gastrointestinal tumors (N = 173)^a

Variable	Group	N=173 (%)	Median OS (months)	HR (95% CI) Univariate ^d	P value Univariate	HR (95% CI) Multivariate ^e	P value Multivariate ^e
Sex	Women	78 (45%)	19.7	Reference Group	0.025	Reference Group	0.01
	Men	95 (55%)	10.0	1.76 (1.08-2.88)		1.87 (1.13-3.06)	
Age	≤60 years	93 (54%)	21.3	Reference Group	0.015	Reference Group	0.02
	>60 years	80 (46%)	10.3	1.83 (1.13-2.97)		1.78 (1.09-2.90)	
(Total Alterations x 100 / kbp) ^b	<1.46	75 (43%)	38.9	Reference Group	1.3x10 ⁻⁶	Reference Group	2.4x10 ⁻⁴
	≥1.46	98 (57%)	6.4	3.46 (2.09-5.72)		3.23 (1.73-6.03)	
(VUS x 100 / kbp) ^b	<0.73	84 (49%)	21.3	Reference Group	2.3x10 ⁻⁶	Omitted ^f	
	≥0.73	89 (51%)	5.0	3.1 (1.95-5.02)			
%ctDNA ^c	<0.5	86 (50%)	21.3	Reference Group	3.8x10 ⁻⁴	Reference Group	0.34
	≥0.5	87 (50%)	9.3	2.46 (1.50-4.03)		1.36 (0.73-2.51)	

Note: All survival analyses were performed from the time of ctDNA collection

Abbreviations: CI = confidence interval; CNS = central nervous system; ctDNA = circulating tumor DNA; %ctDNA = percent circulating tumor DNA; HR = hazard ratio; kbp = kilobase pairs; NR = not reached; OS = overall survival; VUS = variants of unknown significance

^a Patients treated with immunotherapy were excluded

^b Dichotomization done at medians

^c Dichotomization done at medians of highest %ctDNA for each patient; alteration with highest %ctDNA was calculated from all alterations including VUSs. Patients with only amplifications were considered inevaluable and excluded. Kbp indicates the length of ctDNA sequenced: (See **Methods**)

^d Hazard Ratio performed using log-rank

^e Variables with $P \leq 0.05$ in univariate analysis were included in the multivariate analysis. Separate analyses were done to include %ctDNA, total alterations

^f VUS was excluded from the multivariate analysis because it is encompassed within total alterations

Figure 1: Overall survival from ctDNA collection according to %ctDNA (n=418). Low to intermediate %ctDNA was dichotomized at the median of 0.4%. Intermediate to high %ctDNA was dichotomized at 5% because it had been found to be significant in prior studies [10]. The %ctDNA for each patient was calculated using the alteration with the highest allele fraction, including VUSs.

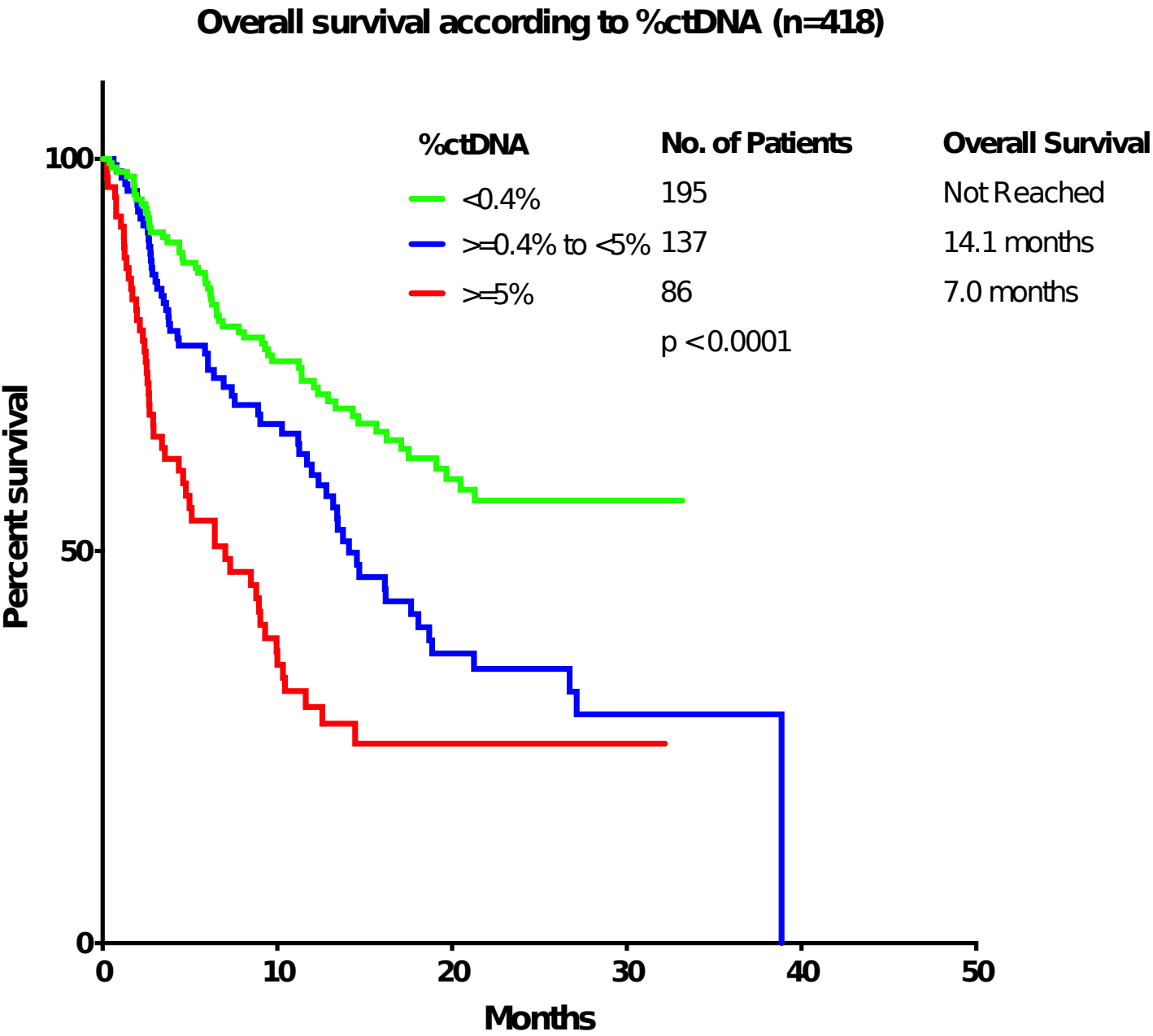


Figure 2: Overall survival from ctDNA collection according to total alterations, including VUS (n=418). Low to intermediate number of alterations was dichotomized at the median of 1.46 alterations.

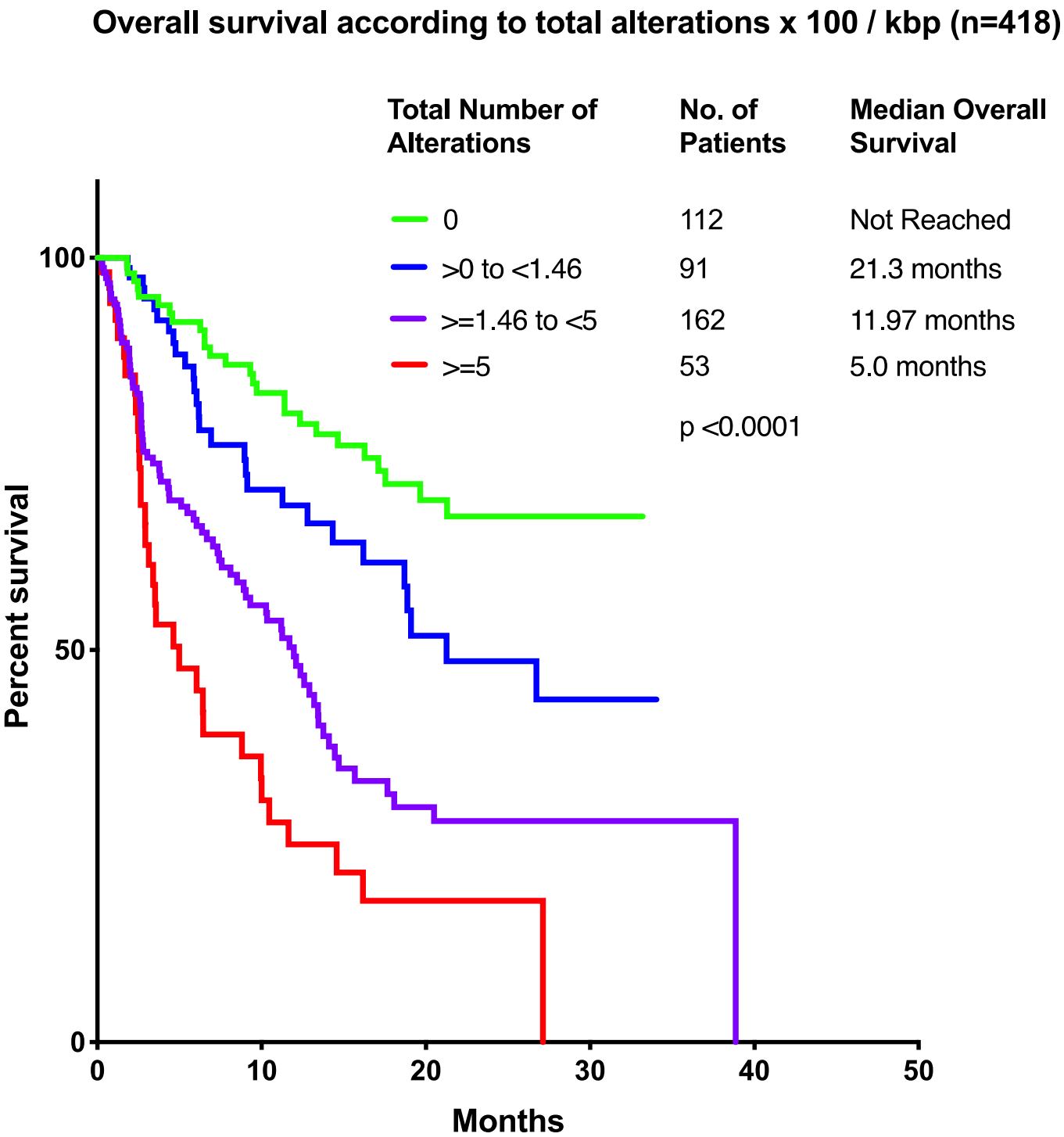
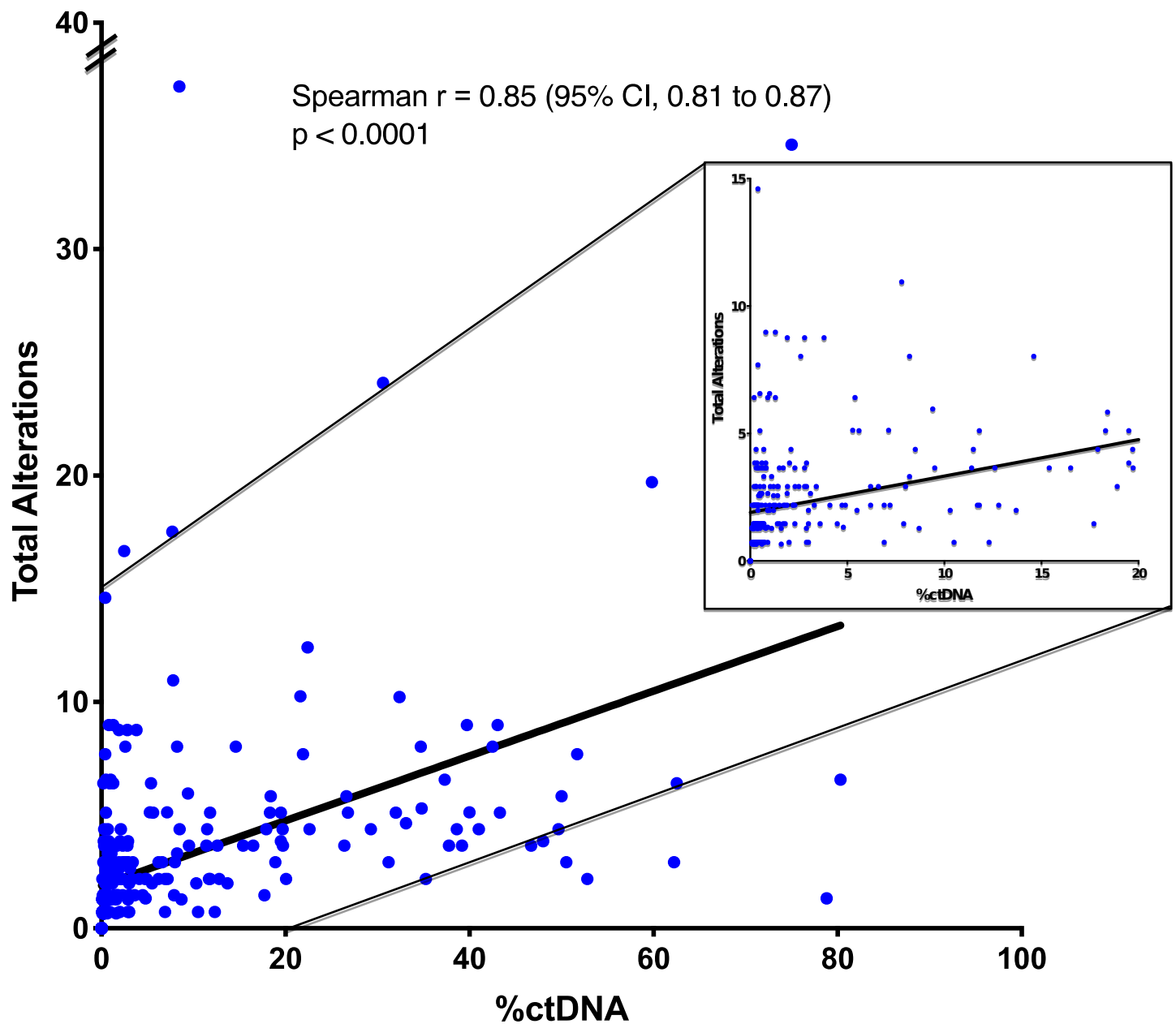


Figure 3: Spearman correlation between %ctDNA and total number of alterations (n=418).

Correlation between %ctDNA and total alterations x 100 / kbp (n=418)



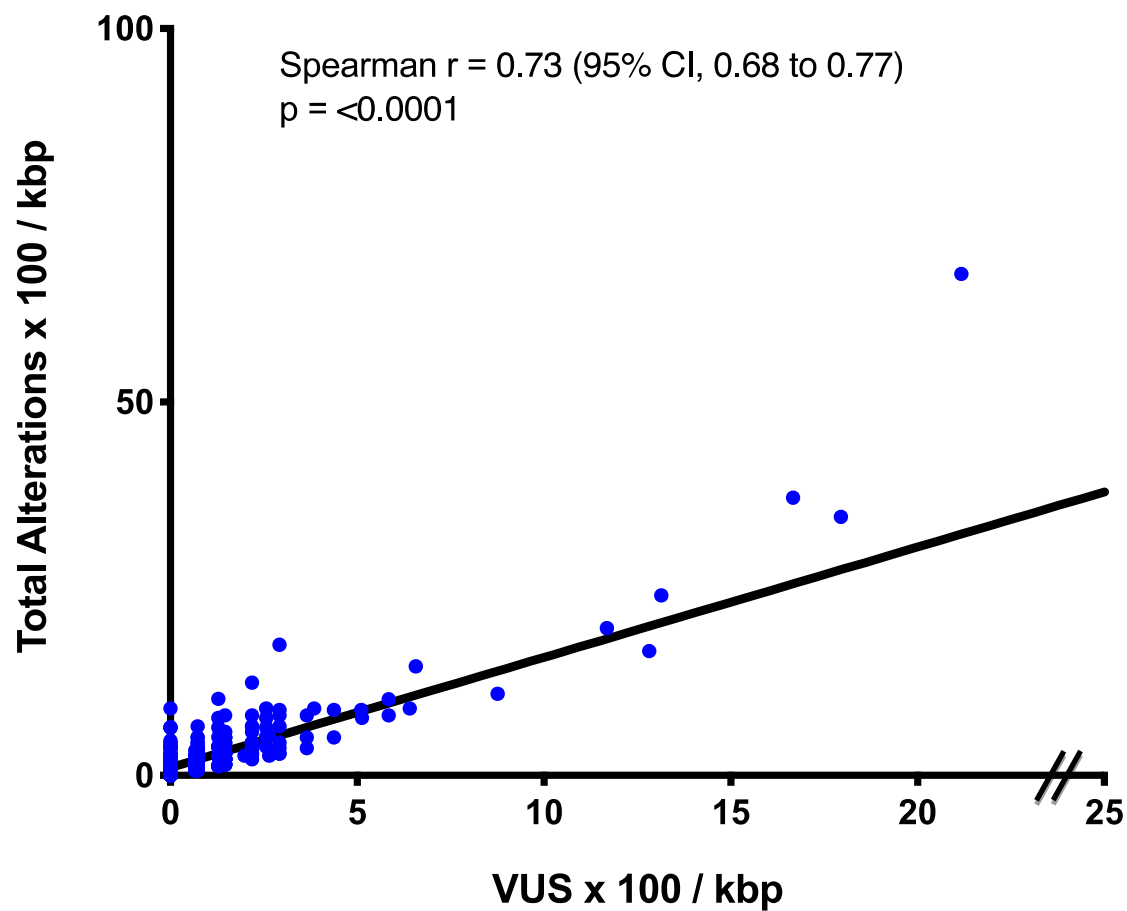
Supplemental Table 1: Guardant360 ctDNA NGS panel and length of DNA sequenced.

Number of Patients Analyzed	Number of Genes	Panel Start Date	Size
47	54	2014	78 kbp
140	68	February 2015	137 kbp
174	70	October 2015	137 kbp
56	73	November 2016	151 kbp
1	73	September 2017	167 kbp*

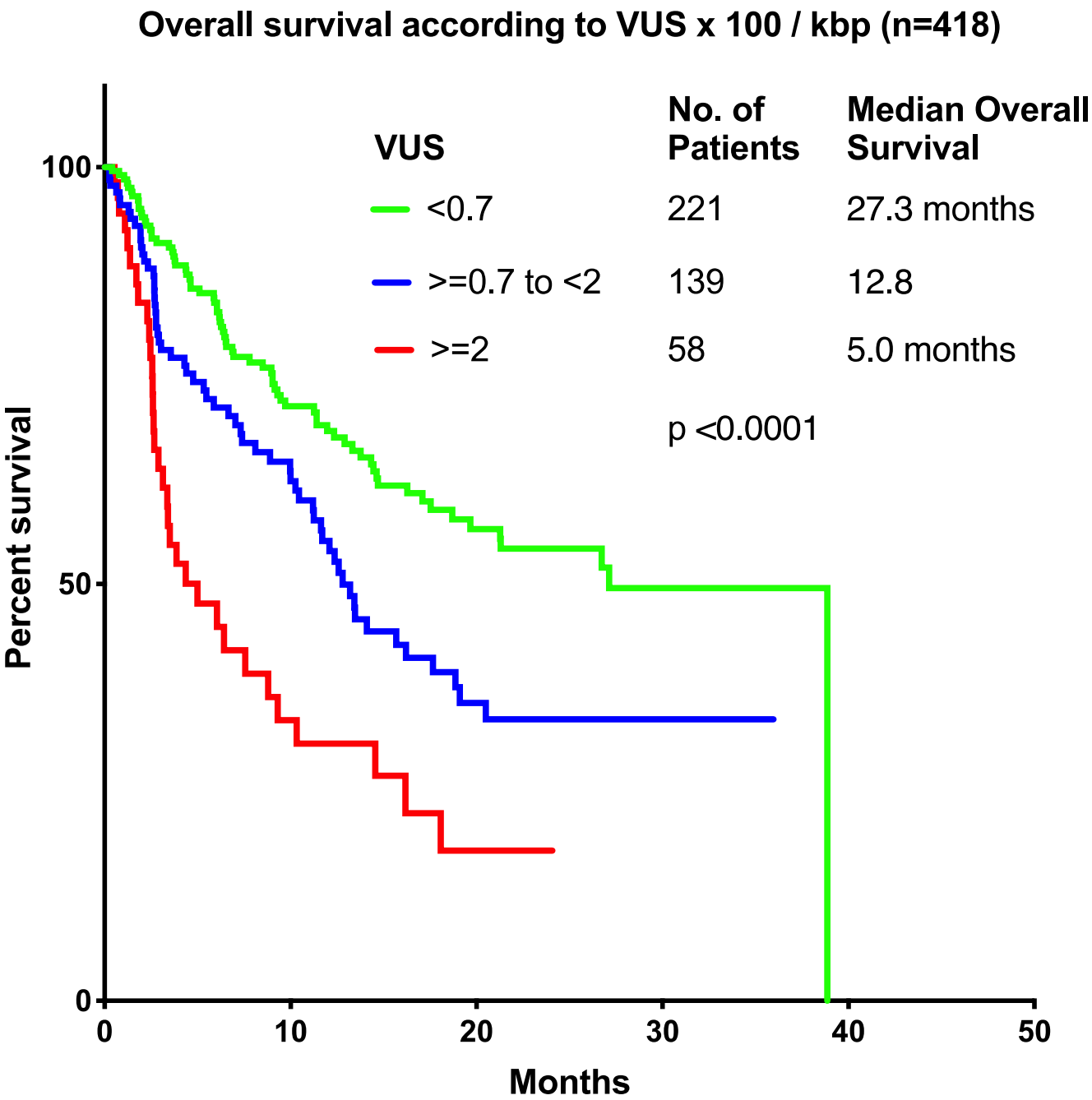
*Version 2 of the 73 gene panel with no changes to reportable results

Supplemental Figure 1: Spearman correlation between VUS and total alterations (n=418).

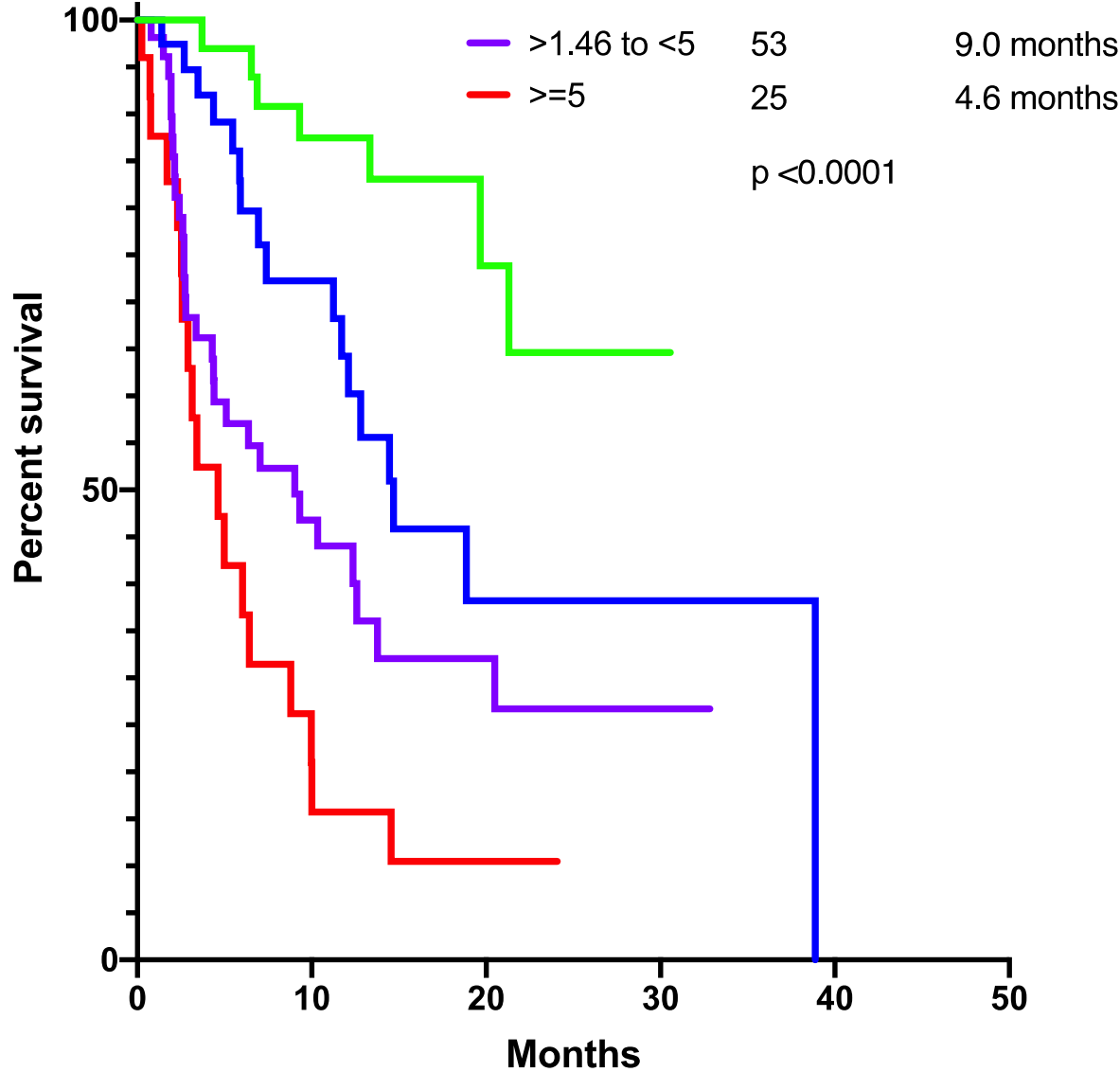
Correlation between VUS x 100 / kbp and total alterations x 100 / kbp (n=418)



Supplemental Figure 2: Overall survival from the date of ctDNA collection according to VUS (n=418). Dichotomization for low to intermediate number of alterations was done at the median of 0.7.



Supplemental Figure 3: Overall survival from ctDNA collection according to total alterations, including VUS (n=173), for the gastrointestinal patients only. Low to intermediate number of alterations was dichotomized at the median of 1.46.



Supplemental Figure 4: Overall survival from ctDNA collection according to %ctDNA (n=173). Low to intermediate %ctDNA was dichotomized at the median of 0.5%. Intermediate to high %ctDNA was dichotomized at 5% because it had been found to be significant in prior studies [10]. The %ctDNA for each patient was calculated using the alteration with the highest allele fraction, including VUSs.

Overall survival in GI patients according to %ctDNA (n=173)

